Claim 2 has been amended to clarify that the recited 22 amino acid residues are in the "portion of CFTR" recited in claim 1.

Claim 7 has been amended to specify that the portion of CFTR protein consists of the amino acid residues shown in SEQ ID NO: 2. The amendment is supported by the specification which discloses that the CFTR protein is activated by a polypeptide that "consists of a portion of CFTR protein which comprises 22 amino acids as shown in SEQ ID NO: 2." (Page 3, lines 23-24.) Original claim 2 also supports this amendment.

Claims 1, 2, and 7 have been amended to correct the terminology of polypeptide constituents from "amino acids" to "amino acid residues."

None of these amendments introduce new matter.

Objections to the Specification

The specification has been objected to because it contains embedded hyperlinks and/or other forms of browser-executable code. Applicants have amended the specification such that all hyperlinks are removed, and replaced them with descriptions of the websites to which those hyperlinks refer.

The drawings have also been objected to for being mislabeled. A set of corrected, formal drawings accompany this response. Withdrawal of these objections is respectfully requested.

The Rejection of Claim 7 Under 35 U.S.C. § 112

Claim 7 has been rejected under 35 U.S.C. § 112 as not being enabled. Applicants respectfully traverse.

In order to satisfy the enablement requirement of 35 U.S.C § 112 the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without undue experimentation. *In re Wright*, 999 F.2d 1557 (Fed. Cir. 1993).

The Office Action alleges that claim 7 is not enabled because it is unclear how to use an unphosphorylated CFTR polypeptide without undue experimentation. The Office Action questions how the unphosphorylated polypeptide is useful because the specification discloses that the art teaches that unphosphorylated R domain inhibits CFTR chloride channels while the object of the invention is to enhance the open probability of CFTR. (Paper 10, page 4, lines 3-12.)

Amended claim 7 recites that the portion of the CFTR protein consists of a sequence of amino acid residues as shown in SEQ ID NO: 2. SEQ ID NO: 2 (amino acid residues 817-838 of CFTR) does not contain an amino acid residue that is phosphorylated. See Ma. (*News Physiol. Sci. 15*, 2000, 154-158; Exhibit 1.) Ma teaches that the R domain contains six PKA phosphorylation sites and two PKC phosphorylation sites. "R domain (amino acids 590-859) contains multiple consensus PKA-phosphorylation sites, 6 of which are used in vivo (660, 700, 737, 768, 795, 813)." (Figure 1 legend, page 155, lines 4-5.) "[T]here are two PKC consensus sites in the R domain (S686 and S790)." (Page 155, column 2, lines 36-37.) Thus the amino acid residues of SEQ ID NO: 2 (817-838) are not phosphorylated.

The specification demonstrates that the NEG2 polypeptide (amino acid residues 817-838; SEQ ID NO: 2) stimulates CFTR protein. (Example 4; page 11, line 13 through page 12, line 7.) Thus the portion of the CFTR protein consisting of a sequence of amino acid residues as shown in SEQ ID NO: 2 that is free of phosphorylation stimulates the activity of the CFTR channel.

This teaching and data rebut the assertion that unphosphorylated polypeptides are inhibitory. Thus one of skill in the art would not have to resort to undue experimentation to practice the invention recited in claim 7. Based on the data supplied in the specification one would expect the claimed polypeptides to be stimulatory. Withdrawal of this rejection to claim 7 is respectfully requested.

The Rejection of Claims 1 and 2 Under 35 U.S.C. § 102(e)

Claims 1 and 2 have been rejected under 35 U.S.C. § 102(e) as being anticipated by Tsui *et al.*, U.S. Patent 5,776,677. Applicants respectfully traverse.

Claim 1 is directed to an isolated polypeptide comprising a portion of CFTR protein. The portion of CFTR protein consists of between 18 and 100 amino acid residues and comprises 18 amino acid residues as shown in SEQ ID NO: 1. Claim 2 further recites that the portion of the CFTR protein comprises 22 amino acid residues as shown in SEQ ID NO: 2.

To reject claims as anticipated, each element of the claims must be found in a single prior art reference. "A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987). Tsui does not teach every element as set forth in amended claims 1 and 2.

Tsui is cited for teaching a CFTR protein that comprises the amino acid sequence shown in SEQ ID NO: 1 and SEQ ID NO: 2. (Paper 10, page 5, lines 16-18.) Tsui teaches the full-length, 1480 amino acid residue sequence of the CFTR protein. (See SEQ ID NO: 16.) Claim 1, however, recites a portion of the CFTR protein. The portion "consists of between 18 and 100

amino acid residues" that comprise the 18 amino acid residues as shown in SEQ ID NO: 1. Tsui does not teach a polypeptide comprising a portion of the CFTR protein consisting of 18 to 100 amino acid residues and having the sequence shown in SEQ ID NO 1. Thus Tsui does not each and every element of rejected claim 1. Withdrawal of this rejection to claim 1 is respectfully requested.

Claim 2 is dependent on claim 1 and thus incorporates all the elements of claim 1. Thus Tsui also does not teach all elements of clam 2. The rejection of claim 2 should also be withdrawn.

The Rejection of Claims 3-6 Under 35 U.S.C. § 103(a)

Claims 3-6 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Tsui *et al.*, U.S. Patent 5,776,677, in view of Welsh *et al.*, WO 95/25796, and Langel *et al.*, U.S. Patent 6,025,140. Applicants respectfully traverse.

Claim 3 is directed to an isolated polypeptide. The polypeptide comprises a portion of CFTR protein. The portion of CFTR protein consists of between 18 and 100 amino acid residues and comprises 18 amino acid residues as shown in SEQ ID NO: 1. The polypeptide is fused to a membrane-penetrating peptide. Claim 4 is directed to an isolated polypeptide like claim 3 with the further requirement that the portion comprises 22 amino acid residues as shown in SEQ ID NO: 2. Claims 5 and 6 recite that the membrane-penetrating peptide of claims 3 and 4 is selected from the group consisting of: VP-22 (SEQ ID NO: 3), (SEQ ID NO: 4), and (SEQ ID NO: 5).

To reject a claim as *prima facie* obvious three criteria must be met:

> First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to

> one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations.

MPEP § 1243. The Patent Office has failed to make a *prima facie* case of obviousness because the combination of Tsui, Welsh, and Langel does not teach or suggest all the limitations recited in claims 3-6.

Tsui is cited for teaching a cystic fibrosis protein comprising the amino acid sequences in SEQ ID NO: 1 and SEQ ID NO: 2. (Paper 10, page 6, lines 7-9.) Welsh is cited for teaching that truncated CTFR polypeptide can be administered alone or in association with an agent that facilitates passage through cell membranes. (Paper 10, page 6, lines 13-15.) Langel is cited for teaching membrane-penetrating peptide sequences that are identical to SEQ ID NO: 4 and SEQ ID NO: 5 of the instant application. (Paper 10, page 6, lines 18-20.) However, none of Tsui, Welsh, or Langel teaches or suggests a portion of CFTR protein that "consists of between 18 and 100 amino acid residues" and that comprises the 18 amino acid residues as shown in SEQ ID NO: 1 or the 22 amino acid residues as shown in SEQ ID NO: 2. This portion has the surprising ability to stimulate CFTR protein chloride channels, even when not phosphorylated.

Tsui, discussed above, teaches the full-length 1480 amino acid sequence of human CFTR polypeptide. Tsui does not teach or suggestion a portion of CFTR that consists of between 18 and 100 amino acid residues and that comprises the 18 amino acid residues as shown in SEQ ID NO: 1 or the 22 amino acid residues as shown in SEQ ID NO: 2.

Welsh does not remedy the defect of Tsui. Welsh teaches truncated CFTR polypeptides. The "truncated CFTR polypeptide molecules comprise a chloride ion channel and a regulator of

the opening and closing of the channel." (Page 14 of Welsh, lines 7-14.) The regulator domain of the CFTR polypeptide "regulates anion passage through the Cl^- channel, is encoded by exon 13 of the genomic CFTR gene, and includes a 241 amino acid sequence spanning from about amino acid residue 590 to residue 830 of full length CFTR." (Page 19 of Welsh, lines 9-11.) Thus Welsh teaches a truncated CFTR polypeptide that comprises at least 241 amino acids of the CFTR protein. Welsh does not teach or suggest a portion of CFTR that "consists of between 18 and 100 amino acid residues" and that comprises the 18 amino acid residues as shown in SEQ ID NO: 1 (amino acid residues 817-834) or the 22 amino acid residues as shown in SEQ ID NO: 2 (amino acid residues 817-838).

Langel also does not remedy the defect of Tsui. Langel teaches peptides and nucleic acid analogs that are used to transport molecules across lipid membranes. Langel does not teach CFTR polypeptides or portions of CFTR polypeptides. Thus Langel also fails to teach or suggest a portion of CFTR that "consists of between 18 and 100 amino acid residues" and that comprises the 18 amino acid residues as shown in SEQ ID NO: 1 or the 22 amino acid residues as shown in SEQ ID NO: 2.

Because none of Tsui, Welsh, and Langel teach the recited portions of CFTR protein the combination of Tsui, Welsh, and Langel fails to teach or suggest all elements of claims 3-6. The Patent Office has thus failed to make a *prima case* of obviousness with respect to claims 3-6. Withdrawal of this rejection to claims 3-6 is respectfully requested.

Respectfully submitted,

Date: August 7, 2002

By: 

Michelle L. Holmes-Son
Registration No. 47,660

Banner & Witcoff, Ltd.
1001 G Street, N.W., Eleventh Floor
Washington, D.C. 20001-4597
(202) 508-9100

Appendix 1. Marked up Version of the Claims and Specification to Show the Changes Made.

Claims

1. (Amended) An isolated polypeptide comprising a portion of CFTR (cystic fibrosis transmembrane conductance regulator) protein wherein said portion consists of between [10] 18 and 100 amino acid residues [acids], wherein said portion [comprising] comprises 18 amino acid residues [acids] as shown in SEQ ID NO: 1.

2. (Amended) The polypeptide of claim 1 [which] wherein the portion of CFTR protein comprises 22 amino acid residues [acids] as shown in SEQ ID NO: 2.

7. (Amended) The polypeptide of claim 1, wherein the portion of CFTR protein consists of a sequence of amino acid residues as shown in SEQ ID NO: 2, and wherein the portion [which] is free of phosphorylation.

Specification

The paragraph at page 8, lines 5-14.

It is believed that the administration of the polypeptide of the present invention will be the most useful in treatment of a class of mutants which produce CFTR proteins which are properly delivered to the plasma membrane but which are only residually or minimally active. Known mutants of CFTR are listed at [http://www.genet.sickkids.on.ca/cftr-cgi-bin/fulltable] the following URL address: http file type, www host server, domain name genet.sickkids.on.ca, directory cftr-cgi-bin, subdirectory fulltable. One can determine that a particular CFTR mutant is fully processed and reaches the plasma membrane in a Western blot assay using antibody against CFTR. Fully processed mutants achieve mature glycosylation status and appear on the

gel as "band C and band B" whereas mutants that are retained in the endoplasmic reticulum are not fully glycosylated and show only "band B". See Example 2, below and Figure 1C.

The paragraph at beginning at page 9, line 18 and ending at page 10, line 12.

The R domain of CFTR contains two negatively charged regions, amino acids 725-733 (NEG1) and amino acids 817-838 (NEG2), that reside in close proximity to two PKA phosphorylation sites, S737 and S813, used in vivo (Figure 1A) (Cheng, et al. 1991). NEG2 is predicted to form an amphipathic (-helical structure with a negatively charged face (Figure 1B) (Geourjon and Deleage, 1995, Rost and Sander, 1993, Rost and Sander, 1994). Three mutations (E822K, E826K, D836Y), two of which were clearly obtained from patients with CF (E822K and D836Y), have been identified within the NEG2 region that result in the removal of negative charges [(www.genet.sickkids.on.ca)] (See URL address: www host server at domain name genet.sickkids.on.ca). The E822K CFTR channel has a low open probability relative to wt-CFTR (wild type-CFTR), but the E826K CFTR channel has single channel properties similar to wt-CFTR (Vankeerberghen et al., 1998). The presence of these disease-causing mutations suggests the potential importance of the NEG2 region. To investigate the roles of NEG1 and NEG2 in CFTR function, these regions were deleted from CFTR using mutagenesis and subcloning. The ΔNEG1- and ΔNEG2-CFTR proteins were transiently expressed in human embryonic kidney 293 cells. Membrane vesicles containing the CFTR proteins were isolated and subjected to SDS-PAGE. Like wt-CFTR, both ΔNEG1- and ΔNEG2-CFTR are present both in the core glycosylated (band B) and the fully glycosylated form (band C) (Figure 1C).

Stimulatory and Inhibitory Functions of the R Domain on CFTR Chloride Channel

Jianjie Ma

CFTR is a chloride channel whose gating process involves coordinated interactions among the regulatory (R) domain and the nucleotide-binding folds (NBFs). Protein kinase A phosphorylation of serine residues renders the R domain from inhibitory to stimulatory and enables ATP binding and hydrolysis at the NBFs, which in turn control opening and closing of the chloride channel.

In 1989, the gene responsible for cystic fibrosis was isolated, and the protein product of this gene was named cystic fibrosis transmembrane conductance regulator (CFTR; Ref. 12). The amino acid sequence of CFTR was used to predict a structure, which consists of two sets of six membrane-spanning domains (MSDs), two nucleotide-binding folds (NBFs), and an intracellular regulatory (R) domain (Fig. 1*A*). This structure is similar to that of the ATP-binding cassette (ABC) family of transporters, but the R domain is unique to CFTR. Research during the last decade has identified CFTR as a multifunctional protein, which provides the pore of a linear conductance chloride channel and also functions to regulate other membrane proteins. Mutations in CFTR leading to defective regulation or transport of chloride ions across the apical surface of epithelial cells are the primary cause of the genetic disease cystic fibrosis (15).

As a chloride channel, CFTR is regulated by two cytosolic pathways. For the channel to open, the protein must first be phosphorylated by a cAMP-dependent protein kinase (PKA), and then intracellular ATP must bind to the NBFs and subsequently be hydrolyzed. The use of compounds that alter the ATP hydrolysis cycle of CFTR, such as AMP-PNP, pyrophosphate, and vandate, provides evidence that hydrolysis of ATP is required for both channel opening and closing transitions. Structure-function studies have suggested that ATP hydrolysis at NBF1 is responsible for opening of the chloride channel and that ATP hydrolysis at NBF2 terminates a burst of open events (5). Since cells normally contain high intracellular levels of ATP, regulation in the intact cell of CFTR is by phosphorylation. The R domain contains the consensus phosphorylation sites for PKA that are the basis for physiological regulation of channel opening (12).

Patch-clamp technique vs. lipid bilayer reconstitution of CFTR channel

The CFTR chloride channels are located in the apical membrane of epithelial cells. To study the function and regulation of the CFTR channel, two electrophysiological methods are commonly used: the patch-clamp technique and lipid bilayer reconstitution. These studies are carried out with either the primary or immortal cultures of epithelial cells expressing the endogenous CFTR proteins or the heterologous cell lines that either stably or transiently express

J. Ma is an Associate Professor in the Department of Physiology and Biophysics at Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, OH 44106.

0886-1714/99 5.00

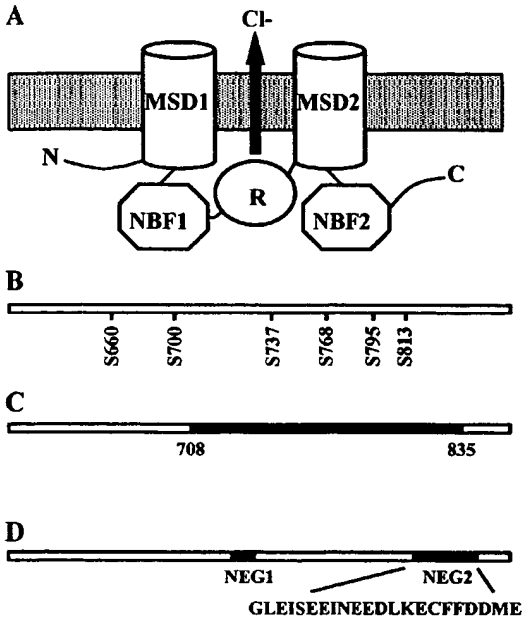

FIGURE 1. Unique feature of R domain of CFTR. *A*: CFTR contains 2 motifs, each containing a membrane-spanning domain (MSD) and a nucleotide-binding fold (NBF), that are linked by a large intracellular regulatory (R) domain. *B*: R domain (amino acids 590–859) contains multiple consensus PKA-phosphorylation sites, 6 of which are used in vivo (660, 700, 737, 768, 795, 813). *C*: residues 708–835 are unique to CFTR and have no homologues in other members of ATP-binding cassette family. *D*: two regions of R domain contain a high proportion of negatively charged amino acids: NEG1 (725–733) and NEG2 (817–838). Of the 22 amino acids in NEG2, 10 are negatively charged. NEG2 is predicted to form an amphipathic α-helical structure with negatively charged residues facing 1 side of helix.

the recombinant CFTR proteins. With both methods, a small current (~10^{-12} A) reflecting the movement of chloride ions through an aqueous pore formed by the CFTR molecule is measured under a given electrochemical potential, and the channel opening and closing transitions are monitored at various physiological conditions (i.e., PKA phosphorylation, ATP binding and hydrolysis, and so forth). Compared with the patch-clamp technique, the bilayer reconstitution system has several advantages. First, the lipid bilayer is formed across two aqueous compartments of milliliter-size dimensions. With this system, it is easy to change the ionic composition of both intracellular and extracellular solutions and to add modulators to either side of the CFTR channel (Ref. 8; see also Figs. 3 and 4). Second, reconstitution methods are easily implemented. Bear et al. (2) used lipid bilayer reconstitution to demonstrate conclusively that the purified CFTR protein is capable of functioning as a PKA- and ATP-dependent chloride channel. The reconstitution system also allows the study of CFTR proteins that are synthesized and retained in the endoplasmic reticulum membranes, i.e., processing mutants of CFTR such as ΔF508 and N1303K (15). These misprocessed CFTR channels are inaccessible to the patch-clamp electrode. Third, the system allows control of membrane phospholipid composition. For example, various surface charge densities and different thicknesses of the bilayer membrane can be achieved by changing the lipid compositions, which can be used to study the effect of membrane structure on the gating and conduction properties of the CFTR channel. The bilayer reconstitution system, however, has one major disadvantage compared with the patch-clamp technique, which is the excess amount of electrical noise associated with the large capacitance of the bilayer membrane. Thus, to enhance the signal-to-noise ratio, one would need to remove the high-frequency noises by using a low-pass cutoff filter. This does not seem to be a problem, since the CFTR channel has a characteristic slow open-close gating process (2, 5, 8).

Multiple phosphorylation sites in the R domain

Ten consensus phosphorylation sites for PKA and two for protein kinase C (PKC) are identified in the R domain, and only six of these sites seem to be heavily used in the intact CFTR molecule (Fig. 1*B*). These sites appear to be redundant and to have additive effects on CFTR function, since mutation of any one of them, or up to three of them, does not affect the maximal activation of the CFTR channel (11). But mutation of more than four major sites reduces the activation by PKA and the open probability of the channel (3). Phosphorylation apparently increases channel openings by adding negative charges, because the substitution of negatively charged amino acids like aspartate for the serines at the consensus sites results in an open channel without phosphorylation, provided that more than six residues are so substituted (11). On the other hand, not all phosphorylation sites are equivalent. Some are inhibitory, whereas others are stimulatory (13). S737 and S768 appear to be inhibitory sites for the channel because mutations S737A and S768A increase the basal activity of the CFTR channel. Even CFTR with all 10 consensus phosphorylation sites for PKA mutated maintains residual PKA-dependent openings (3), suggesting that other cryptic phosphorylation sites (either inside or outside the R domain) also contribute to the overall function of the CFTR channel.

Although there are two PKC consensus sites in the R domain (S686 and S790) and phosphorylation of these sites occurs in vivo and in vitro, PKC stimulation alone does not activate the CFTR channel. Prephosphorylation by PKC seems to be essential for the PKA-dependent activation of the CFTR channel since it potentiates the effect of PKA, but once CFTR has been fully activated by PKA channel activity is not increased by application of PKC (7).

Effects of deleting the R domain on CFTR function

Sequence alignment of CFTR with P-glycoprotein and other members of the ABC transporter family reveals that CFTR contains an extra 128 amino acids in the R domain (residues 708–835; Fig. 1*C*). Deletion of this portion of the R domain from CFTR, ΔR(708–835), removes the requirement for PKA phosphorylation to open the CFTR channel (10). Unlike the wild-type (wt) CFTR, which opens in a strictly PKA-dependent manner, the ΔR(708–835) channel opens without PKA phosphorylation, and, furthermore, its open probability does not change with PKA phosphorylation (Fig. 2*A*). The ΔR(708–835)

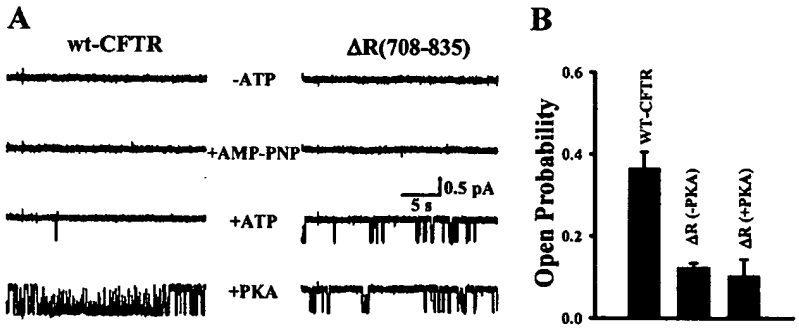

FIGURE 2. PKA dependence of wild-type (wt) and ΔR(708–835) CFTR channels. *A*: experimental records from a single CFTR channel reconstituted into lipid bilayer membrane. Opening of wt CFTR absolutely requires hydrolysis of ATP and PKA phosphorylation (*left*). Without ATP (–ATP) or in the presence of nonhydrolyzable analog of ATP (+AMP-PNP), the channel does not open. In the absence of PKA, ATP alone (+ATP) is insufficient to induce channel opening. Channel openings are visible only when both ATP and PKA (+PKA) are present in the intracellular solution. Partial deletion of R domain, ΔR(708–835), results in a PKA-independent CFTR channel that still requires hydrolyzable ATP but has fewer openings than wt CFTR (*right*). Downward deflections represent movement of chloride ions from intracellular to extracellular solutions. With 200 mM chloride ions as current carrier, both wt CFTR and ΔR(708–835) channels exhibit a linear conductance of ~8 pS. *B*: average open probability (P_o) of the ΔR(708–835) channel [ΔR(–PKA)] is ~1/3 that of wt CFTR channel. P_o of ΔR(708–835) channel does not change with PKA phosphorylation [ΔR(+PKA)].

channel also exhibits altered response to AMP-PNP and pyrophosphate, compounds that prolong burst duration of the wt CFTR channel but fail to do so in the ΔR(708–835) channel (9). On the basis of these studies, it has been proposed that this portion of the CFTR molecule contains the putative gating particle of the chloride channel (10).

Compared with the wt CFTR, open probability of the ΔR(708–835) channel is significantly lower (Fig. 2*B*). This suggests that some stimulatory property of the R domain has been lost in the ΔR(708–835) mutant or that removal of these 128 amino acids from the R domain introduces structural changes that could affect the function of the NBFs.

Interaction of exogenous R domain peptide with the CFTR channel

Further insights into the function of the R domain in the CFTR channel are obtained through reconstitution studies of the interaction of the exogenous R domain protein with a single CFTR channel captured in the lipid bilayer or in excised membrane patches. The R domain peptide (amino acids 590–858) interacts specifically with the wt CFTR channel in a phosphorylation-dependent manner (8). When applied to the intracellular side of the channel, the unphosphorylated R domain peptide inhibits opening of the wt CFTR channel, but once the R domain peptide is phosphorylated, the inhibitory effect is relieved and the channel reopens (Fig. 3*A*). Interestingly, addition of the unphosphorylated R domain peptide has no effect on the ΔR(708–835) channel. However, when the exogenous R domain protein is phosphorylated, significant stimulation of the ΔR(708–835) channel occurs (Ref. 9 and 14; Fig. 3*B*). The results indicate that the R domain does not function solely as an inhibitor that keeps the channel closed, so it is not simply an "on-off" switch for the channel. The function of the R domain differs mechanistically from the "ball-and-chain" model for the *Shaker* potassium channel, in which the amino-terminal "ball" is thought to physically obstruct the ion conduction pathway for potassium ions (6).

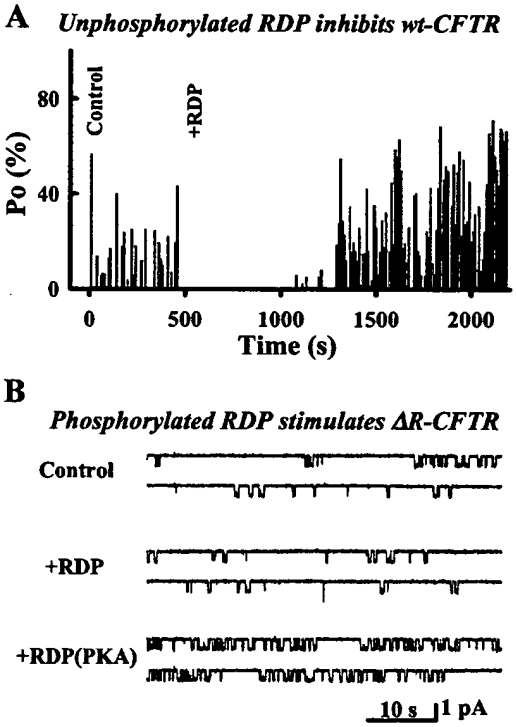

FIGURE 3. Effect of exogenous R domain protein on CFTR channel. *A*: phosphorylation-dependent blocking of wt CFTR channel by exogenous R domain protein (RDP, amino acids 590–858). P_o of a single wt CFTR channel was measured with 2 mM ATP and 50 U/ml of catalytic subunit of PKA present in cytosolic solution (Control). Addition of RDP to cytosolic solution resulted in transient inhibition of channel, followed by spontaneous recovery of channel openings (+RDP). This recovery of channel activity was due to phosphorylation of RDP by PKA present in cytosolic solution, because pretreatment of channel with a peptide inhibitor of PKA, which prevented phosphorylation of RDP, led to permanent closure of channel by RDP (not shown). Thus only unphosphorylated RDP is capable of blocking wt CFTR channel. *B*: stimulation of ΔR(708–835) channel by phosphorylated R domain protein. Selected current traces from ΔR(708–835) channel show that same RDP peptide, when unphosphorylated, had no effect on ΔR(708–835) channel. But when phosphorylated by PKA [+RDP(PKA)], RDP stimulated ΔR(708–835) channel.

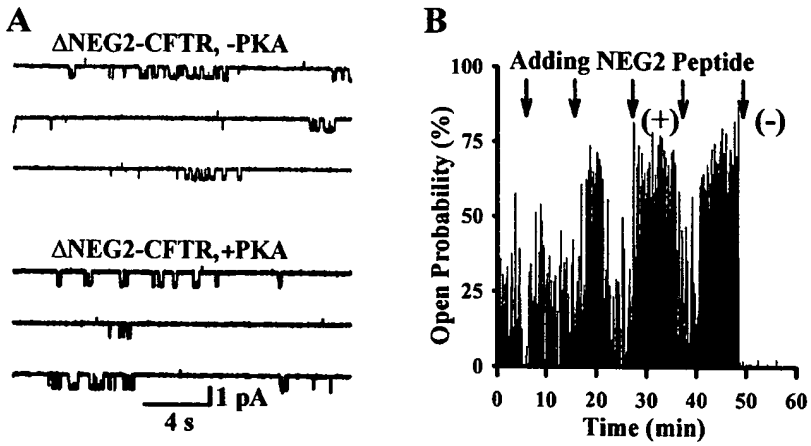

FIGURE 4. Stimulatory and inhibitory functions of NEG2 on CFTR channel. *A*: effects of deleting NEG2 region (amino acids 817–838) on CFTR function. ΔNEG2-CFTR channel opens without requiring PKA phosphorylation (–PKA). This is similar to ΔR(708–835) channel but in contrast to wt CFTR channel (see Fig. 2). On addition of PKA (100 U/ml) to cytosolic solution, activity of ΔNEG2-CFTR channel does not change. *B*: stimulatory and inhibitory effects of NEG2 peptide on CFTR channel. After addition of NEG2 peptide to cytosolic solution, there are periods of intense stimulation (+) of wt CFTR channel, followed by either a return to basal level of activity or by an almost complete inhibition of channel (–). Arrows indicate addition of NEG2 peptide at concentrations of 0.9–18 μM.

The data suggest the following putative model for CFTR function. CFTR forms a chloride channel in which parts of the transmembrane domains constitute the pore and the R domain functions as a channel inhibitor until it is phosphorylated by PKA and undergoes a conformational change to make the pore accessible to chloride ions. Phosphorylation of the R domain has two effects on CFTR: the first could be permissive, releasing a steric hindrance on the channel; the second might be stimulatory, facilitating interaction of ATP with the NBFs. Once the R domain is phosphorylated, binding and hydrolysis of ATP take place at NBF1, which leads to opening of the chloride channel. Subsequent binding and hydrolysis of ATP at NBF2 closes the channel. The closed state of the channel can be secured by dephosphorylation of the R domain.

Stimulatory and inhibitory functions of a short segment of the R domain

The R domain of CFTR contains two negatively charged regions, amino acids 725–733 (NEG1) and amino acids 817–838 (NEG2), that reside in close proximity to two PKA phosphorylation sites, S737 and S813, used in vivo (Fig. 1*D*). Two cystic fibrosis-associated mutations have been identified within the NEG2 region that result in the removal of negative charges, E822K and D836Y. The presence of these disease-causing mutations suggests the potential importance of the NEG2 region in CFTR function. Deletion of NEG1 from the R domain has no significant impact on the CFTR channel in terms of ion permeation and PKA-dependent gating. But deletion of NEG2 from the R domain produces a functional ΔNEG2-CFTR channel that opens without PKA, with open probability ~1/5 that of the wt CFTR (Fig. 4*A*). Moreover, addition of PKA up to 200 U/ml, four times the concentration required to fully activate the wt CFTR channel (8), does not increase the open probability of the ΔNEG2-CFTR channel (1). Thus removal of NEG2 from CFTR completely eliminates the PKA dependence of the chloride channel, although the ΔNEG2-CFTR still contains all 10 PKA phosphorylation sites. The synthetic 22-amino acid NEG2 peptide interacts with the CFTR molecule and exhibits both stimulatory and inhibitory effects on CFTR function (Fig. 4*B*). Additionally, covalent modification of a cystine residue at *position 832*, which resides within NEG2, by *N*-ethylmaleimide results in irreversible stimulation of PKA-phosphorylated CFTR channel activity, further emphasizing the importance of NEG2 in CFTR function (4).

These data show that the NEG2 region could confer both stimulatory and inhibitory functions of the R domain on the CFTR channel. When this region is deleted from CFTR, the resultant channel opens without PKA (loss of inhibitory function), but it never achieves open probability comparable with wt CFTR even when phosphorylated with PKA (loss of stimulatory function). This same NEG2 sequence, expressed as a peptide, results in stimulation of channel openings at lower concentrations and profound inhibition of channel activity at higher concentrations when added to the intracellular side of the CFTR channel. It seems likely that this sequence could interact with CFTR at different sites on the NBFs to either stimulate or inhibit channel openings.

Conclusion

The fact that the R domain has both stimulatory and inhibitory roles in CFTR channel function has important implications for the pharmacological and gene-therapeutic interventions of cystic fibrosis. First of all, understanding how the R domain works in the CFTR channel, i.e., by identifying the stimulatory interaction of NEG2, may facilitate the design of therapeutic reagents that stimulate CFTR opening to treat patients whose mutant forms of CFTR reach the cell surface. Furthermore, because gene therapy for cystic fibrosis has been plagued by inefficient delivery of the gene and inefficient expression of CFTR once the gene has been delivered, it would be desirable to have a form of CFTR that retains the absolute

requirement for PKA activation (i.e., is regulated normally) and has increased open probability when fully activated. Since the conformation of the R domain is an important determinant of whether it functions in a stimulatory or inhibitory mode, and if the conformation of the R domain can be changed to enhance its activator function in the phosphorylated state without altering its inhibitory activity in the unphosphorylated state, the open probability of the phosphorylated channel might be increased while the channel remains under strict control of ATP and PKA. The wt CFTR channel, when fully activated, has an average open probability of ~0.30 (Fig. 2*B*). Therefore, there is potential to improve the overall function of CFTR to achieve a better vehicle for gene therapy of cystic fibrosis, either by mutating the R domain or the NBFs or by improving the intramolecular interactions among the three hydrophilic domains of CFTR: NBF1, R, and NBF2.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-51770 and an Established Investigatorship from the American Heart Association to J. Ma.

References

1. Adams, L. M., and J. Ma. Deletion of a negatively charged region (a.a. 817–838) from the R domain of CFTR alters PKA-dependent regulation of the CFTR channel (Abstract). *Biophys. J.* 74: A344, 1998.
2. Bear, C. E., C. Li, N. Kartner, R. J. Bridges, T. J. Jensen, M. Ramjeesingh, and J. R. Riordan. Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). *Cell* 68: 809–818, 1992.
3. Chang, X. B., J. A. Tabcharani, Y. X. Hou, T. J. Jensen, N. Kartner, N. Alon, J. W. Hanrahan, and J. R. Riordan. Protein kinase still activates CFTR chloride channel after mutagenesis of all 10 PKA consensus phosphorylation sites. *J. Biol. Chem.* 268: 11304–11311, 1993.
4. Cotten, J. F., and M. J. Welsh. Covalent modification of the regulatory domain irreversibly stimulates cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 272: 25617–25622, 1997.
5. Gadsby, D. C., and A. C. Nairn. Regulation of CFTR channel gating. *Trends Biochem. Sci.* 19: 513–518, 1994.
6. Hoshi, T., W. N. Zagotta, and R. W. Aldrich. Biophysical and molecular mechanisms of *Shaker* potassium channel inactivation. *Science* 250: 533–538, 1990.
7. Jia, Y. L., C. J. Mathews, and J. W. Hanrahan. Phosphorylation by protein kinase C is required for acute activation of cystic fibrosis transmembrane conductance regulator by protein kinase A. *J. Biol. Chem.* 272: 4978–4984, 1997.
8. Ma, J., J. Tasch, T. Tao, J. Zhao, J. Xie, M. L. Drumm, and P. B. Davis. Phosphorylation dependent block of cystic fibrosis transmembrane conductance regulator chloride channel by exogenous R domain protein. *J. Biol. Chem.* 271: 7351–7356, 1995.
9. Ma, J., J. Zhao, M. L. Drumm, J. Xie, and P. B. Davis. Function of the R domain in the cystic fibrosis transmembrane conductance regulator chloride channel. *J. Biol. Chem.* 272: 28133–28141, 1997.
10. Rich. D. P., R. J. Gregory, M. P. Anderson, P. Manavalan, A. E. Smith, and M. J. Welsh. Effect of deleting the R domain on CFTR-generated chloride channels. *Science* 253: 205–207, 1991.
11. Rich, D. P., H. A. Berger, S. H. Cheng, S. M. Travis, M. Saxena, A. E. Smith, and M. J. Welsh. Regulation of the cystic fibrosis transmembrane conductance regulator chloride channel by negative charge in the R domain. *J. Biol. Chem.* 268: 20259–20267, 1993.
12. Riordan, J. R., J. M. Rommens, B.-S. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J.-L. Chou, M. L. Drumm, M. C. Iannuzzi, F. S. Collins, and L.-C. Tsui. Identification of the cystic fibrois gene: cloning and characterization of complementary DNA. *Science* 245: 1066–1073, 1989.
13. Wilkinson, D. J., T. V. Strong, M. K. Mansoura, D. L. Wood, S. S. Smith, F. S. Collins, and D. C. Dawson. CFTR activation: additive effects of stimulatory and inhibitory phosphorylation sites in the R domain. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 273: L127–L133, 1997.
14. Winter, M. C., and M. J. Welsh. Stimulation of CFTR activity by its phosphorylated R domain. *Nature.* 389: 294–296, 1997.
15. Zielenski, J., and L.-C. Tsui. Cystic fibrosis: genotypic and phenotypic variations. *Annu. Rev. Genet.* 29: 777–807, 1995.